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FOREWORD

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David Klever
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INTRODUCTION

The treatment of prostate cancer has largely centered on the inhibition of testosterone production. This is effected mainly by castration, or through the use of drugs such as LHRH agonists. We propose that other hormonal factors also play a role in the growth and development of the prostate and prostatic cancers. Specifically, we have strong experimental evidence that IGF-I, a mediator of GH action, is a very effective stimulator of the glandular development of mouse ventral prostate. We propose to extend these observations to better understand the mechanism of action of IGF-I, and whether similar effects might be observed in prostate cancer. Our goals are to answer the following questions: Is IGF-I necessary for testosterone action? Can the effect(s) of IGF-I be localized to either the stromal or epithelial compartment of the prostate? Is IGF-I important in the maintenance of prostate gland structures once they are fully formed? Can IGF-I stimulate human prostate cancers in xenografts in nude mice? Can inhibitors of IGF-I action inhibit prostate tumor growth?

BODY

Tasks 1 and 2.

We feel that we have made significant progress in achieving some of the goals of these 2 tasks. A paper of our findings has been published in *Endocrinology*, a copy of which is included in the appendix. We found that prostate glands from 44 day old IGF-I knockout animals were not only smaller than their wild-type counterparts, but also had fewer terminal duct tips and degree of branching. These differences were highly significant ($p < 0.0001$). Administration of des(1-3)-IGF-I for 7 days partially reversed these deficits. Similarly, mice transgenic for a GH antagonist had impaired prostate development as determined by histological analysis. These data suggest that GH plays an important role in the development of the prostate. However, since GH has no independent effect on stimulation of the prostate in the IGF-I knockouts, it probably works through IGF-I as an intermediary.

Part of the goal of these 2 tasks was to assess the effects of GH or IGF-I on prostate development in "little" mice, which lack GHRH and are thus deficient in GH and IGF-I. When compared with lit/+ heterozygotes, ventral prostates from lit/lit animals had significantly reduced numbers of terminal ductal tips (67 ± 1.3 vs 48.6 ± 1.1), quaternary branch points (49 ± 1.2 vs 37 ± 1.2), and glandular area (cm^2) (1.76 ± 0.08 vs 0.71 ± 0.02). All differences were highly significant by Students t-test ($p < 0.0001$). Administration of bGH to lit/lit animals by miniosmotic pumps for 7 days, restored levels near to those of lit/+ animals: terminal ductal tips = 61 ± 1 ; quaternary branch points = 46 ± 1.5 ; glandular area = 1.12 ± 0.09 . Similar effects were observed in the dorsal prostates of these animals.

We have also done preliminary experiments examining the effect of leuprolide, an LHRH agonist, on the ability of IGF-I to stimulate prostate development in IGF-I knockout animals. This study is important because it helps to determine whether the IGF-I effect on prostate development is independent of testosterone, or whether testosterone is required for an IGF-I effect. We found that when leuprolide was given simultaneously with des (1-3)-IGF-I to the knockout animals, there was a complete inhibition of the IGF-I effect: Terminal duct tips in the untreated animals numbered 23.0 ± 2.1 which were increased to 37.0 ± 2.6 after 28 days treatment with the des(1-3)-IGF-I; however, simultaneous treatment with IGF-I + leuprolide yielded a value of 23.0 ± 0.9 . Similarly, the area of the ventral prostate increased from a control value of 0.27 ± 0.01 to 0.73 ± 0.09 following IGF-I treatment. In contrast, treatment with both IGF-I and leuprolide yielded an area of 0.28 ± 0.02 , essentially identical to control animals. Leuprolide alone had no apparent effect on these parameters. These results strongly imply that testosterone and IGF-I act in synergy in promoting prostate development.

Task 3.

We have only recently begun to do the experiments associated with this task. We have established a solution hybridization/Rnase protection assay for both IGF-I receptor and IGF-I mRNAs, but do not yet have any data on quantitation or localization. Similarly, the methodology for immunohistochemistry of both IGF-I and its receptor are in place, but no data is yet available.

Tasks 4 and 5.

The experiments proposed for these tasks have not yet begun. They are scheduled to begin after the first 18 months of funding.

KEY RESEARCH ACCOMPLISHMENTS

- The demonstration that IGF-I and GH are of major importance in the development of the mouse prostate gland.
- Preliminary data suggesting that IGF-I acts in synergy with testosterone to promote the development of the mouse prostate gland.

REPORTABLE OUTCOMES

The research funded by this grant resulted in a publication in the journal *Endocrinology*. It is entitled "Evidence that insulin-like growth factor I and growth hormone are required for prostate gland development" 140:1984-1989, 1999.

CONCLUSIONS

The results presented in this progress report are potentially of great importance to our understanding of normal prostate growth, and by inference to prostate cancer as well. While a great deal of effort has been spent on elucidating the role of testosterone in the etiology and progression of prostate cancer, only moderate gains have been achieved clinically. Our results using mouse models of prostate development have shown that IGF-I is of major importance in this regard, and might act in synergy with testosterone in the formation of terminal ducts and branch points. It is therefore plausible that a similar synergy might be involved in the growth of prostate cancers. It is our hope that the results presented here might be a catalyst for the development of IGF-I inhibitors or antagonistic drugs. The use of these drugs might prove of efficacy when used in combination with testosterone lowering drugs in the treatment of prostate cancer.

REFERENCES

Ruan, W., Powell-Braxton, L. Kopchick, J.J., and Kleinberg, D.L. Evidence that insulin-like growth factor I and growth hormone are required for prostate gland development. *Endocrinology* 140:1984-1989, 1999.

APPENDICES

3 reprints attached

Evidence That Insulin-Like Growth Factor I and Growth Hormone Are Required for Prostate Gland Development*

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ABSTRACT

Insulin-like growth factor I (IGF-I) has been implicated as a factor that may predispose one to prostate cancer. However, no specific relationship between IGF-I and prostate development or cancer *in vivo* has been established. To determine whether IGF-I was important in prostate development, we examined prostate architecture in IGF-I^{-/-} null mice and wild-type littermates. Glands from 44-day-old IGF-I-deficient animals were not only smaller than those from wild-type mice, but also had fewer terminal duct tips and branch points and deficits in tertiary and quaternary branching ($P < 0.0001$), indicating a specific impairment in gland structure. Administration of des(1-

3)-IGF-I for 7 days partially reversed the deficit by increasing those parameters of prostate development ($P < 0.006$). That IGF-I production probably mediates an effect of GH in this process was indicated by the observations that GH antagonist transgenic mice also had significantly impaired prostate development ($P < 0.0002$) and that bovine GH had no independent effect on stimulating prostate development in IGF-I null animals. The data indicate that IGF-I deficiency is the proximate cause of impaired prostate development and give credence to the idea that, like testosterone, GH and IGF-I may be involved in prostate cancer growth as an extension of a normal process. (*Endocrinology* 140: 1984-1989, 1999)

RECENT epidemiological evidence supports the possibility that insulin-like growth factor I (IGF-I) may play a role in either stimulating development or fostering growth of prostate cancer. A positive association between serum concentrations of IGF-I and prostate cancer risk was reported by several groups (1-3). A natural question is whether this supposed effect of IGF-I on prostate cancer is specific, like that of testosterone (T), which affects both the growth and development of the normal prostate and prostate cancer (4, 5), or whether it is nonspecific. Although prostate cancer cell growth is stimulated by IGF-I in culture, there has been little direct evidence that IGF-I has anything at all to do with the development or integrity of the normal prostate gland or the pathogenesis or furtherance of prostate cancer. Given the similarities between the mammary gland and the prostate (6) and our previous observations that GH and probably IGF-I were essential for mammary gland development (7-11), we undertook studies to determine whether the control of prostate development was similar to the control of mammary gland development. To determine whether GH and IGF-I were also essential for prostate development, we studied two animal models in which prostate development would have been expected to be deficient if our hypothesis was correct, an IGF-I^{-/-} null mouse model (12, 13) and a transgenic mouse expressing a GH antagonist (14-18).

Materials and Methods

Animals

IGF-I^{-/-} null animals were bred in our laboratory. Male mice that were heterozygous (-/+) for IGF-I (raised by L.B.-P.) were mated with 8-week-old CD1 female mice (Charles River Laboratories, Inc., Wilmington, MA) to provide heterozygous animals for mating. One heterozygous male was placed in a cage with one or two heterozygous females. Although it was expected that 25% of the animals would be completely devoid of the gene for IGF-I, only approximately 1-2% survived. Of those, 66% were males, and 34% were females. Wild-type animals weighed a mean of 31 g vs. 6.2 g in the knockouts ($P < 0.0001$). The animals were housed in sterile cages, and sterile technique was used for their handling. The presence or absence of the IGF-I gene was confirmed by PCR on DNA extracted from mouse tails as previously described (12, 13).

Heterozygous transgenic mice carrying the gene for a GH antagonist, as previously reported (14-18), were also raised in our laboratory. We crossed these males with C57BL female mice (Charles River Laboratories, Inc.). As expected, 25% of the offspring were heterozygous for this transgene. Fifty percent were female, and 50% were male. In addition to these transgenic animals being smaller (0.57 times) than wild-type animals ($P < 0.0001$), they were identified by PCR on tail snips, as previously described.

Examination of the prostate gland

Prostate glands were removed from 44-day-old male mice under tribromoethanol/amylen hydrate anesthesia. After decapitation and collection of blood, prostate glands were removed *en bloc*, including the seminal vesicles, coagulating glands, urethra, and bladder. The prostate was freed from the other structures. To determine glandular detail, separation of glandular structures from stromal ones was carried out according to a modification of the method of Sugimura *et al.* (19). Periprostatic fat was removed by careful dissection in calcium- and magnesium-free Hanks' solution. Thereafter, the prostates were transferred to calcium- and magnesium-free Hanks' solution with 1% collagenase for 5-10 min at room temperature to digest the connective tissue and permit a clearer view of glandular structures of the prostate. This was followed by further dissection under a Nikon dissecting microscope

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(SMZ-U, Melville, NY) at $\times 20$ magnification. Fine forceps and 27-gauge needles were employed for further dissection. At the end of this procedure, the prostate lobes from which connective tissue had been removed were photographed (magnification, $\times 3.75$) so that glandular structures could be examined, counted, and compared. Both the ventral and dorsal prostates were examined. The following parameters were analyzed: area of the gland, number of terminal duct tips, primary, secondary, tertiary and quaternary branching, and number of branch points.

Treatment with hormones

Hormone treatment was begun at 37 days of age, so that examination of the prostate could be carried out at 44 days. IGF-I (20 μ g; a gift from Genentech, Inc., South San Francisco, CA) and bovine GH (bGH; 100 μ g; a gift from Monsanto Corp., St. Louis, MO) were administered by Alzet model 1007D miniosmotic pumps (Alza Corp., Palo Alto, CA). These pumps were designed to deliver hormone or growth factor over a period of 7 days at 0.5 μ l/h. T (Calbiochem, La Jolla, CA) was given in a SILASTIC brand capsule (Dow Corning Corp., Midland, MI) implanted sc, as previously described (8). Serum T concentrations at the end of experiments revealed that concentrations in null animals treated with T for 7 days were similar to those in untreated wild-type littermates (range, 509–653 ng/dl). T was measured by RIA using reagents from ICN (Costa Mesa, CA). Serum IGF-I in knockout animals treated with des(1–3)-IGF-I, assayed by a Nichols Institute Diagnostics 100T kit (San Juan Capistrano, CA), ranged from 36–90 ng/ml, whereas control animals had unmeasurable levels (<30 ng/ml). Under anesthesia, 37-day-old mice had SILASTIC capsules and Alzet miniosmotic pumps containing hormone, growth factor, or vehicle in controls implanted sc. After 7 days, animals were killed, prostates were removed, and blood was collected. Prostate glands were then examined as described above.

Results

Prostate development in GH antagonist transgenic mice

To determine whether IGF-I in prostate gland development was under the control of GH, we examined prostate development in 44-day-old transgenic male mice expressing a bovine GH antagonist and compared it to development in wild-type littermates. This GH antagonist binds to the GH receptor, inhibits the action of endogenous GH, and causes dwarfism in the animals carrying this transgene (14–18). Our hypothesis was that development would have been impaired in this animal model if GH played a role in prostate development. As shown in Table 1 and Fig. 1, prostate development was significantly impaired with regard to the number of terminal duct tips in both the dorsal ($P < 0.0002$) and ventral ($P < 0.0001$) lobes and also in the two-dimensional area of the prostate glands ($P < 0.0001$). In the ventral prostate there was also a significant reduction in the number of branch points in transgenic *vs.* wild-type animals (24.8 *vs.* 47; $P < 0.0001$) and in the number of quaternary branches (16.6 *vs.* 40; $P < 0.0001$).

Prostate development in IGF-I-deficient mice

To determine whether the small size of the prostate gland in IGF-I-deficient mice, as previously noted by Baker *et al.* (13), was due merely to a reduction in overall gland size or to the combination of reduced gland size and a more specific abnormality in the developmental process, we compared prostate gland architecture in 44-day-old IGF-I^{-/-} male mice (these animals do not make IGF-I) with that in wild-type littermates that produce normal amounts of IGF-I.

As shown in Table 2 and Fig. 2, the area of the ventral prostate lobes was more than 10 times greater in prostates

TABLE 1. Comparison of prostate development of bGH antagonist transgenic dwarf mice and wild-type litter mates

| | Ventral prostate | | | | | Dorsal prostate | | |
|-------------------------------|-----------------------------|------------------------------|---------------|---------------|----------------|-----------------------------|-----------------------------|---------------|
| | Terminal duct tips | Area (cm ²) | 1° Branching | 2° Branching | 3° Branching | Branch points | Terminal duct tips | Main ducts |
| Wild-type (n = 5) | 61.0 \pm 1.5 | 1.39 \pm 0.12 | 2.2 \pm 0.2 | 5.4 \pm 0.2 | 27.0 \pm 2.4 | 47.0 \pm 0.7 | 48.0 \pm 3.3 | 9.6 \pm 0.5 |
| bGH mutant transgenic (n = 5) | 35.4 \pm 1.7 ^a | 0.38 \pm 0.04 ^a | 2.2 \pm 0.2 | 5.4 \pm 0.2 | 23.8 \pm 0.4 | 24.8 \pm 1.2 ^a | 23.6 \pm 1.0 ^a | 8.4 \pm 0.2 |

^a Significantly different from wild-type, $P < 0.0002$.

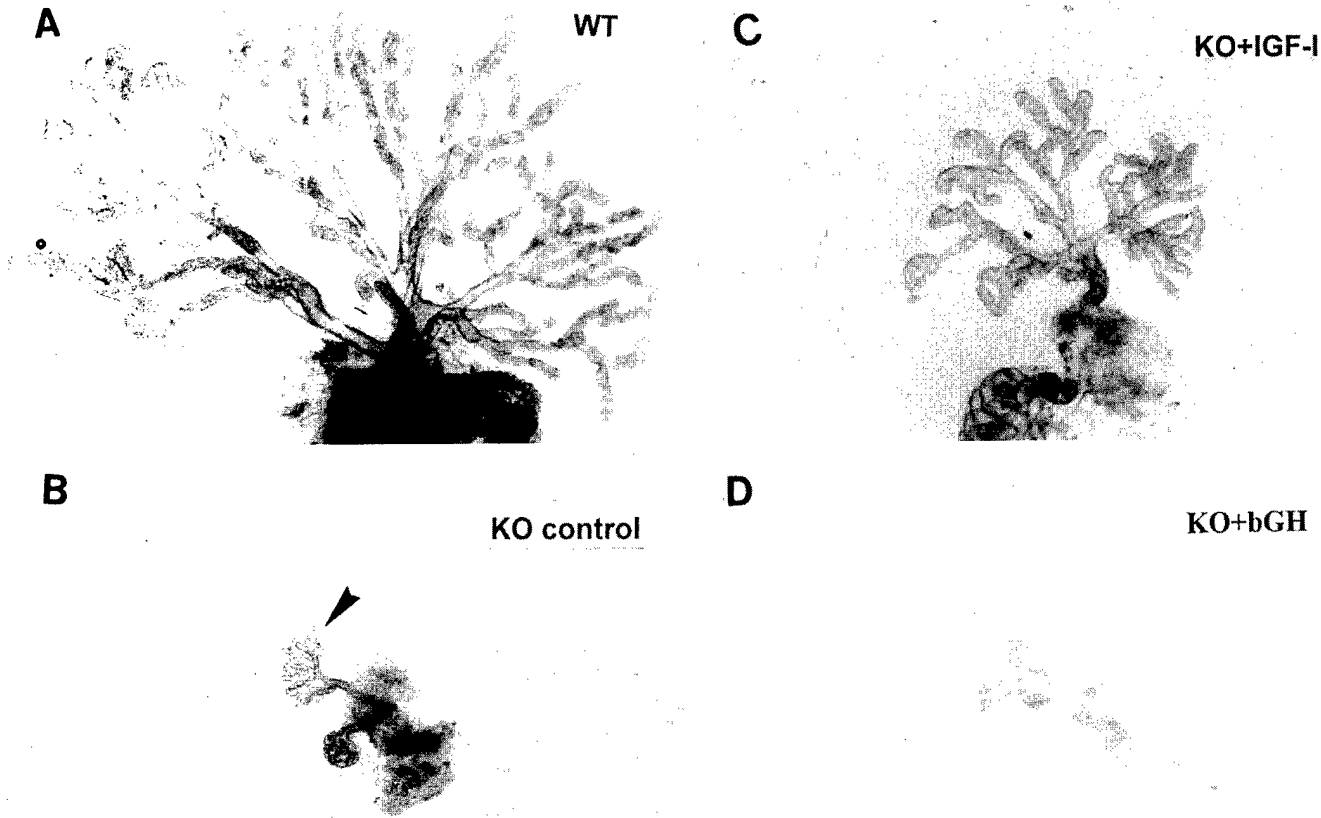


FIG. 2. Photomicrographs of representative ventral prostate glands from 44-day-old wild-type or IGF-I^{-/-} null mice. A, Wild-type 44-day-old male mouse; B, an untreated IGF-I^{-/-} knockout mouse; (C), an IGF-I^{-/-} null animal treated with des(1-3) IGF-120 μ g for 7 days; and (D), an IGF-I^{-/-} null animal treated with bGH100 μ g for 7 days. Note the obvious differences between (A) and (B). An arrowhead points to the ventral prostate of the IGF-I knockout mouse that has been fully dissected (B). The size of the single ventral lobe of the gland above was found to be much larger than to the entire gland from the knockout animal (0.06 vs. 1.86 cm²). Also, there were fewer branch points (12 vs. 40), less tertiary (14 vs. 22) and quaternary branching (4 vs. 42), and fewer terminal duct tips (17 vs. 50) in the IGF-I knockout than in the wild-type animal. When an IGF-I^{-/-} null animal was given des(1-3) IGF-I for 7 days (C), the size of prostate vs. that of the IGF-I knockout control (A) rose from 0.06 to 0.61 cm². There was an increase in the number of branch points (12 vs. 23), tertiary (14 vs. 20) and quaternary (4 vs. 11) branching, and number of terminal duct tips (17 vs. 27). In contrast, treatment with bGH for 7 days had no effect on prostate development (D). All images were photographed at 3.8 \times magnification.

treated IGF-I^{-/-} animals with an amino-terminally shortened form of IGF-I, called des(1-3)-IGF-I. It binds poorly to IGF-binding proteins and is therefore more active than native IGF-I (8, 20). This form of IGF-I was administered alone and also together with T. Other variables included T alone and bGH alone. IGF-I, either without or with T, stimulated significant development of the prostate gland in IGF-I^{-/-} mice compared with controls (Table 2). Increases in the number of terminal ductal tips ($P < 0.001$), area ($P < 0.006$), branch points ($P < 0.0005$), and tertiary ($P < 0.004$) and quaternary ($P < 0.001$) branching were noted in response to IGF-I. Although T alone significantly stimulated these parameters also, the effect of IGF-I alone was greater, and there was no apparent synergy between T and IGF-I noted. A representative photomicrograph of a prostate gland, with detail of the ventral lobe, taken from an animal treated with des(1-3)-IGF-I alone and one taken from a control knockout animal are shown in Fig. 2. Similar observations were made when the

architecture of the dorsal prostate was determined (Table 2). Again, IGF-I significantly increased the size of the prostate ($P < 0.003$) and the number of terminal ductal tips ($P < 0.00001$). Thus, the deficient prostate development found in animals incapable of making IGF-I can at least in part be reversed by treatment with a form of IGF-I for 7 days.

As shown in Table 2 and Fig. 3, administration of bGH had no effect on restoring prostate development in IGF-I-deficient animals. If GH had an independent effect on prostate development that was not mediated by IGF-I, such an effect would have been expected.

Relationship of prostate development to animal size

The degree of prostate development in the two dwarf animal models we employed was also expressed in relation to their size. We expressed body weight and parameters of prostate gland development as a percentage of those in con-

trol animals (Table 3) and then determined means for the entire groups of animals. We found that the numbers of terminal duct tips, tertiary branching, and branch points were appropriate for the sizes of the animals, but that gland area and quaternary branching were significantly more impaired than could have been accounted for by size alone ($P < 0.04$).

Discussion

These data indicate that IGF-I and GH are involved in the normal development of the prostate gland during gestation and in early life. The concept that IGF-I played such a role has been previously suggested by Baker *et al.* and Liu *et al.* (13, 21, 22). They found small prostates in these animals, but the nature of the impairment was not evaluated, nor were studies performed to ascertain whether the actual IGF-I deficiency was responsible. In addition to an overall reduction in prostate size, we found specific deficits in gland structure, including the number of terminal ductal tips, and in tertiary and quaternary branching.

Our data also show that these anatomical defects in prostate development can at least in part be reversed by administration of the missing IGF-I. Thus, the prostate is similar to the mammary gland in its requirement for IGF-I (8, 10, 23). In the mammary gland, IGF-I synergizes with estrogen to achieve full pubertal development. Neither estrogen alone nor IGF-I alone is sufficient (8, 10). Although both T and now IGF-I seem to be required for full development of the prostate, it is difficult to comment on the likelihood that these two hormones act in synergy for full prostate development. The reason for this is that IGF-I^{-/-} animals are exposed to enough T *in utero* to develop prostate glands. Also, knockout animals produce some T at the stage of development at which they were examined, albeit not as much as wild-type controls (13, 21, 22). Therefore, it is possible that the level of T present is sufficient for such synergy. In contrast, we did not find synergy between IGF-I and T in prostate development when these two hormones were simultaneously administered. Thus, it is possible that the requirement for IGF-I may not be as crucial for prostate development as it is for mammary development.

Without GH or IGF-I, no mammary gland development occurs. In the female, the pituitary gland (24–28) and specifically GH (7, 29) are essential for mammary gland development. All indications are that IGF-I mediates the effects of GH in that process (8, 10, 30). Until now we have not known whether prostate development also required GH or GH-induced IGF-I for development. Reiter and colleagues provided important clues that GH may have a role in prostate development, as it stimulates IGF-I messenger RNA and

IGF-I receptor messenger RNA in the prostate (31, 32), and Lostroh and Li have shown an effect of GH on prostate size (33). That the prostate gland structure in IGF-I knockout animals was not restored by bGH is consistent with the concept that prostate development and mammary development are similar when it comes to the cascade of GH-induced IGF-I production. A positive effect of GH on prostate growth in this animal model would have indicated a direct effect of GH on prostate development not mediated by IGF-I. Taken together with the observation that transgenic mice producing a GH antagonist have similarly impaired prostate development as that in IGF-I^{-/-} deficient animals signifies that GH is important in prostate development and that its effect is mediated by IGF-I production.

One might question the possibility that prostate development was impaired because of the small size of the animals rather than because of deficiencies in GH and IGF-I. In fact, we found that some of the parameters of prostate development were appropriate to the size of the animals, and others were significantly more impaired than could be accounted for by size. However, we suggest that the deficits in prostate development were due to deficiencies in GH and IGF-I rather than to size. We make this assertion because prostate development in normal mice is quite advanced at the time of birth and is almost fully developed with regard to the number of prostate structures before puberty. Sugimura *et al.* found that the main ducts of the prostate had already undergone secondary and tertiary branching by the time the animals were born (19). By day 15 of life, 80.7% of the terminal duct tips were already present, as were 76.4% of the branch points. In contrast, our IGF-I^{-/-} animals had only 28% the number of expected terminal duct tips and 26% the number of branch points in their prostates at 44 days of age. Furthermore, the small size of the animals was clearly due to deficits in IGF-I or GH, rather than to some unrelated cause of dwarfism. Thus, we believe that the deficit in prostate development is due to absent IGF-I rather than small animal size.

These data also provide a novel method for studying prostate gland development. Previously, it has been difficult to prove a role for GH in prostate development other than gland weight, because significant prostate development takes place during gestation, and virtually all structures, albeit small, are present before puberty (4, 34, 35). An effect on weight alone might be considered nonspecific, because GH increases fluid retention (36). Thus, special animal models, like those we have employed and perhaps others, will have to be employed to pose additional specific questions as to the hormonal control of prostate development.

A recent report showed that patients whose serum IGF-I concentrations were high normal or elevated were at greater

TABLE 3. Comparison of percentage of body weight and related prostate structures in IGF-I^{-/-} mice (KO) *vs.* wild-type litter mates (Wild), KO treated with IGF-I (IGF-I) *vs.* KO, and bGH antagonist-treated transgenic dwarf mice (Tg) *vs.* wild-type litter mates (Wild)

| | BW (%) | Ventral prostate | | | | | Dorsal prostate | |
|----------|------------|--------------------|-------------------------|--------------|-------------------------|---------------|-----------------------|-------------------------|
| | | Terminal duct tips | Area | 3° Branching | 4° Branching | Branch points | Terminal duct tips | Area |
| KO/Wild | 20.0 ± 1.4 | 28.0 ± 2.3 | 6.5 ± 1.6 ^a | 57.0 ± 4.9 | 8.1 ± 1.6 ^a | 26.0 ± 2.4 | 21 ± 3.1 | 5.7 ± 0.58 ^a |
| IGF-I/KO | 144 ± 14 | 170 ± 19 | 364 ± 68 ^a | 122 ± 32 | 322 ± 76 | 180 ± 14 | 226 ± 32 ^a | 398 ± 42 ^a |
| Tg/Wild | 57.0 ± 1.9 | 58.0 ± 2.9 | 28.0 ± 3.4 ^a | 89.0 ± 5.6 | 42.0 ± 5.5 ^a | 53.0 ± 2.5 | 50 ± 4.3 | 28.0 ± 4.2 ^a |

^a $P < 0.04$.

risk for developing prostate cancer (1). That observation together with the fact that IGF-I is a potent stimulator of prostate cancer cell growth suggest that IGF-I may be involved in the genesis or maintenance of prostate cancer. There is, however, a pressing question as to whether the mitogenic effects on prostate cell growth in culture are specific, as IGF-I is a potent mitogen on most cell lines (37). Normal prostate development requires the interaction of both stromal and epithelial elements (6). Therefore, observations on isolated epithelial cells cannot be interpreted as reflective of what would take place *in vivo*. The present study is the first report that shows that normal development of prostate glandular elements is in part dependent upon IGF-I. It also supports the likelihood that IGF-I production in this process may be due to GH, as transgenic animals expressing a GH antagonist also had deficient prostate development. These observations give further credence to the possibility that GH, through IGF-I, is involved in prostate cancer as an extension of its role in normal development. Such a confirmation will stimulate the development of inhibitors of IGF-I and give an impetus to putting them into clinical trials.

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